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Citation for published version:

Brennecke, J, Kraut, S, Zwadlo, K, Gandhi, SK, Pritchard, D, Templeton, K & Bachmann, T 2017, 'High yield extraction of E. coli RNA from human whole blood', *Journal of Medical Microbiology*.
<https://doi.org/10.1099/jmm.0.000439>

Digital Object Identifier (DOI):

[10.1099/jmm.0.000439](https://doi.org/10.1099/jmm.0.000439)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Journal of Medical Microbiology

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High yield extraction of *E. coli* RNA from human whole blood

Running title: *E. coli* RNA from human whole blood

**Johannes Brennecke¹, Simone Kraut^{1,2}, Klara Zwadlo^{1,2}, Senthil Kumar Gandhi¹,
David Pritchard³, Kate Templeton⁴, Till Bachmann^{1*}**

¹ Division of Infection and Pathway Medicine, University of Edinburgh, Edinburgh,
United Kingdom

² AG Aus- und Weiterbildung, Deutsches Krebsforschungszentrum (DKFZ), Heidelberg,
Germany

³ Axis Shield Diagnostics Ltd., Dundee, United Kingdom

⁴ Royal Infirmary, Edinburgh, United Kingdom

* Corresponding author:

email: till.bachmann@ed.ac.uk

phone: +44 (0) 131 242 9438

Keywords: sepsis, bloodstream infection, RNA extraction, molecular diagnostics, *E. coli*,
whole blood, sample preparation

Word count: 5866

Abbreviations: IST broth, Iso-Sensitest broth; SDV, standard deviation; TE buffer, Tris-
EDTA buffer; RIN, RNA integrity number

Abstract: Studies of bacterial transcriptomics during bloodstream infections are limited to-date because unbiased extraction of bacterial mRNA from whole blood in sufficient quantity and quality has proved challenging. We addressed the key aspect of this issue and investigated approaches for the lysis of the human blood cells and the stabilisation of bacterial RNA in human whole blood samples containing *E. coli* cells. We compared four lysis agents: Triton X-100, saponin, ammonium chloride and the commercial MoLYsis lysis buffer CM for their ability to yield high amounts of undegraded RNA and investigated their impact on the bacterial gene expression. For low cell numbers the best mRNA yields were obtained if the commercial RNeasy Protect reagent was added directly to the blood sample without prior lysis of the blood cells. Using this protocol, significant amounts of human RNA were co-purified but did not have a negative impact on qPCR detection of the bacterial mRNA. Importantly, at low concentrations of bacterial RNA, moderate amounts of co-purified human RNA had a positive effect on the yield of bacterial mRNA. Among the tested lysis agents Triton X-100 was the fastest in lysis and reduced the human mRNA background by three to four orders of magnitude compared to stabilisation without prior lysis. As anticipated, treatment with lysis agents was found to be associated with changes in bacterial gene expression. However, these changes were below 1.5-fold for the genes investigated. This study provides a framework for the choice of the most suitable sample preparation method to study bacterial bloodstream infections.

1. Introduction

Bacterial bloodstream infections are a serious and increasing healthcare problem. (Artero *et al.*, 2010; Lark *et al.*, 2001; Martin *et al.*, 2003) Therefore, there is growing interest in bacterial RNA extracted from human whole blood for diagnostic and research purposes. (Barczak *et al.*, 2012; Graham *et al.*, 2005; Macfarlane & Dahle, 1993) We focussed on *E. coli* because it is the most common pathogen found in bloodstream infections (Opota *et al.*, 2015; Wilson *et al.*, 2011) and due to its frequent use in sepsis research (Bhatty *et al.*, 2012; Machuca *et al.*, 2016; Skorup *et al.*, 2014). The analysis of bacterial gene expression can elucidate the interactions between the pathogen and the host and improves our understanding of infection. Additionally, there is potential for molecular diagnostics as bacterial mRNA can reveal information about the etiologic pathogen as well as its antibiotic susceptibilities. (Barczak *et al.*, 2012) Furthermore, bacterial rRNA can be used for pathogen detection and identification (Fujimori *et al.*, 2010; Matsuda *et al.*, 2007; Sakaguchi *et al.*, 2010) and has an advantage over DNA due to its high copy number (Arfvidsson & Wahlund, 2003; Bremer & Dennis, 2008).

We suspect that the wider use of bacterial RNA as a tool in the study and diagnostics of bloodstream infections has been hampered by the difficulty of extracting target bacterial RNA from whole blood specimen in sufficient quantity and of adequate purity for further analysis. Co-purification of PCR inhibitors such as haemoglobin (Akane *et al.*, 1994), anticoagulants (Yokota *et al.*, 1999), leucocyte DNA (Morata *et al.*, 1998) or IgG (Al-Soud *et al.*, 2000), the large excess of human blood cells (Song *et al.*, 2014) and the presence of nucleases (Tan & Yiap, 2009) are amongst the recognised obstacles in the analysis of bacterial DNA from blood and we expected similar problems with the analysis of bacterial

RNA. Another challenge when investigating bacterial gene expression is the intrinsically short half-life of bacterial mRNAs (Bernstein *et al.*, 2002; Hambræus *et al.*, 2003), which requires stabilisation immediately after the collection of the sample.

Extraction of bacterial rRNA from whole blood has been demonstrated using microfluidic devices (Rogacs *et al.*, 2012) as well as conventional methods (Matsuda *et al.*, 2007). Analysis of bacterial gene expression in whole blood using a variety of other extraction methods has also been reported. In some studies, blood cells were lysed (e.g. with saponin) prior to RNA stabilisation (Hedman *et al.*, 2012), whereas in others bacterial gene expression was stabilised first, followed by blood cell lysis with erythrocyte lysis buffer (Echenique-Rivera *et al.*, 2011; Mereghetti *et al.*, 2008; Del Tordello *et al.*, 2012). In another approach, the use of dedicated blood cell lysis agents was avoided altogether and only a reagent for the stabilisation of the bacterial gene expression was applied. (Barczak *et al.*, 2012; Orihuela *et al.*, 2004)

To our knowledge, a systematic analysis of the RNA yield and quality achieved with the different approaches has not been published to-date but would benefit future studies. In this work, we present the analysis of different methods of blood cell lysis and RNA stabilisation with respect to bacterial RNA yield, integrity and purity. Moreover, we discuss the influence of the lysis agents on the bacterial gene expression and the effect of co-purified human RNA on the quantification of bacterial mRNA. We used four different lysis buffers: (1) ammonium chloride solution (often referred to as red blood cell lysis buffer) is commonly used to remove most of the cellular particles of a whole blood sample and thus facilitates the extraction of bacterial nucleic acids. (Shortman *et al.*, 1972) However, it does not lyse white blood cells, leading to significant co-purification of human DNA and

RNA; (2) the commercial, chaotropic MolYsis buffer CM is used in a kit designed for the extraction of bacterial DNA from human whole blood (Hansen *et al.*, 2009; Mccann & Jordan, 2014) and leads to the lysis of all human blood cells; (3) saponin is known to cause holes in the membranes of mammalian cells (Bangham & Horne, 1962), thus leading to specific lysis of these cells. It has been used for the lysis of blood cells to enable the direct detection of bacteria from blood cultures (Chen *et al.*, 2013; Meex *et al.*, 2012) or to lyse the blood cells for subsequent gene expression studies (Hedman *et al.*, 2012); and finally (4) Triton X-100 which, in combination with a high pH buffer, has been described to be a very effective lysis agent for red and white blood cells, while pathogens remain intact. (BIOCARTIS S.A., 2012; Zelenin *et al.*, 2015)

For the stabilisation of the bacterial RNA and the suppression of the synthesis of new RNA molecules we used the commercially available RNAprotect Bacteria reagent. The main component of this agent is described as tetradecyltrimethylammonium bromide. (QIAGEN Ltd, 2015) We applied it either directly to the blood sample or after lysis of the human blood cells. We also investigated the suitability of the commercial PAXgene reagent for the stabilisation of bacterial gene expression in whole blood specimen without prior lysis of the blood cells. The reagent was originally designed for the stabilisation of human mRNA in whole blood. (Rainen *et al.*, 2002)

2. Methods

Culturing of *E. coli* cells

E. coli CFT073 (Luo *et al.*, 2009; Welch *et al.*, 2002) was used throughout this study. When required, the strain was recovered from a glycerol stock onto LB agar (Fisher Scientific, Loughborough, UK) plates and incubated overnight at 37°C. Overnight liquid cultures were grown in 3 ml of IST broth (Oxoid, Basingstoke, UK) in a Multitron standard shaking incubator (Infors, Basel, Switzerland) (37°C; 200 rpm). The next day, 50 µl of the 10⁻⁴ dilution (≈10⁴ c.f.u.) were added to 3 ml of fresh IST broth in a 15 ml reaction tube. The culture was grown for 5 h to mid-exponential phase (37°C; 200 rpm). Bacterial cells from 500 µl of the culture (≈2x10⁷ c.f.u.) were harvested by 3 min of centrifugation at 5000x g in a 5415 D tabletop centrifuge (Eppendorf, Hamburg, Germany). The supernatant was discarded and the pellet was resuspended in 500 µl of human whole blood (anticoagulant: EDTA; Seralab, West Sussex, UK) or fresh IST broth. For experiments with low cell numbers 25 µl of a 10⁻² dilution (≈10⁴ c.f.u.) of the mid-logarithmic culture were spiked directly into 500 µl of whole blood or fresh IST broth.

Stabilisation of bacterial RNA

Four lysis buffers for human whole blood, used for the removal of human cells in the process of bacterial DNA extraction, were tested for their suitability for bacterial RNA extraction. Volumes, concentrations and pH values were as suggested by the manufacturer or as used in the literature. Incubation times were kept the same for all lysis agents to ensure comparability. To lyse blood cells, the blood was mixed with either (1) 500 µl of 1 % [v/v] Triton X-100 (Sigma-Aldrich, Gillingham, UK) in cold 500 mM

carbonate buffer, pH 10.0 (Sigma-Aldrich, Gillingham, UK), (2) 500 µl of 5 % [w/v] saponin (Sigma-Aldrich, Gillingham, UK) in cold PBS buffer (Fisher Scientific, Loughborough, UK), (3) 500 µl of 0.83 % [w/v] NH₄Cl in cold 0.01 M Tris-HCl buffer, pH 7.5 (Sigma-Aldrich, Gillingham, UK) or (4) 125 µl of cold MolYsis buffer CM (Molzym, Bremen, Germany). The samples were incubated for a maximum of 30 s and centrifuged at 8000x g for 3 min to recover the bacterial cells. The supernatant was discarded and the bacterial pellet was resuspended in a mixture of 500 µl of IST broth and 1000 µl of RNaprotect Bacteria reagent (Qiagen, Hilden, Germany). If no dedicated lysis agent was applied, 1000 µl of RNaprotect reagent were added directly to the whole blood or the IST broth. Solutions were mixed by inversion, incubated for 15 min at room temperature, then centrifuged at 6000x g for 6 min. The supernatant was discarded and the remaining pellet was frozen at -80°C until RNA extraction.

Alternatively, blood with bacteria was first stabilised with RNaprotect before adding a lysis agent. After 15 min incubation at room temperature the solution was centrifuged for 6 min at 6000x g. The supernatant was discarded and the pellet was resuspended in a mix of 500 µl of IST broth and one of the lysis buffers described above. The sample was then incubated at room temperature for 5 min on a roller mixer. After centrifugation at 6000x g for 6 min the supernatant was decanted and the pellets were frozen at -80°C until RNA extraction.

For the stabilisation with the PAXgene reagent 500 µl of whole blood with bacteria were mixed with 1.38 ml of PAXgene reagent (PreAnalytiX, Hombrechtikon, Switzerland) and incubated overnight at room temperature. The next day, samples were centrifuged at 5000x g for 10 min. The supernatant was discarded and pellets were washed with 800 µl of nuclease free water (Life Technologies, Carlsbad, CA, USA). Samples were

centrifuged again (5000x g for 10 min) and RNA extraction was performed immediately as described below.

RNA extraction

RNA was extracted using a modified protocol for the RNeasy Mini kit (Qiagen, Hilden, Germany). Pellets were resuspended in 200 µl of TE buffer (Life Technologies, Carlsbad, CA, USA) containing 15 mg/ml lysozyme (Sigma-Aldrich, Gillingham, UK) and 10 µl of proteinase K (Qiagen, Hilden, Germany). Depending on the extent of the lysis of the blood cells the resuspension could be tedious and require time due to the viscosity of the pellet. However, all pellets were resuspended completely. The solution was incubated on a roller mixer for 10 min at room temperature. To lyse the bacterial cells, 700 µl of buffer RLT (kit component) with 10 µl of β-mercaptoethanol (Sigma-Aldrich, Gillingham, UK) were added. After vigorous vortexing the samples were incubated for a further 20 min on the roller mixer. Then, 500 µl of absolute ethanol (VWR, Lutterworth, UK) were added and the solution was mixed vigorously using a vortex mixer. The lysate (≈1.4 ml) was loaded onto an RNeasy spin column in two 700 µl batches by centrifugation at 8000x g for 1 min. The column was washed two times with 350 µl of buffer RW1 (kit component) and two times with 500 µl of buffer RPE (kit component), then transferred into a fresh collection tube and centrifuged at 8000x g for 5 min to remove residual ethanol. Finally, the column was transferred to a new 1.5 ml reaction tube. For elution, 45 µl of nuclease free water were added and the column was centrifuged at 8000x g for 1 min. The flow through was added to the column again and the centrifugation was repeated. The eluate was placed on ice immediately. The amount of extracted nucleic acid was assessed by measurement of the absorption at 260 nm using a NanoDrop ND-1000 spectrophotometer (Thermo

177 Fisher Scientific, Wilmington, DE, USA). The purity of the sample was assessed by
178 measurement of the absorption at 230 and 280 nm.

179 **DNA removal**

180 DNA was removed with the TURBO DNA-free kit (Life Technologies, Carlsbad, CA, USA)
181 according to the manufacturer's instructions. The concentration and purity of the
182 remaining RNA was again assessed with the NanoDrop ND-1000 spectrophotometer.

183 **Measurement of the RNA integrity**

184 RNA integrity was measured by capillary electrophoresis using a 2100 Bioanalyzer
185 instrument (Agilent Technologies, Santa Clara, CA, USA) with the RNA 6000 Nano kit
186 (Agilent Technologies, Santa Clara, CA, USA) following the manufacturer's instructions.

187 **Real-time PCR**

188 From 12 µl of undiluted RNA in a 20 µl reaction cDNA was synthesised using the Tetro
189 cDNA synthesis kit (Bioline, London, United Kingdom) according to the manufacturer's
190 instructions. The final cDNA was diluted if higher volumes were required to allow for the
191 analysis of multiple genes. A master mix was prepared from the SensiFAST SYBR Lo-
192 ROX reagent (Bioline, London, United Kingdom), nuclease free water and the
193 corresponding primers (Metabion, Planegg, Germany) (final concentration: 0.4 µM). The
194 primer sequences are depicted in Table 1.

195 16 µl of mastermix were mixed with 4 µl of cDNA template for a total volume of 20 µl per
196 reaction. PCRs were performed on a Mx3000P instrument (Stratagene-Agilent
197 Technologies, La Jolla, CA, USA) using the following conditions: 2 min initial denaturation

at 95°C, followed by 40 cycles of 5 s at 95°C, 10 s at 60°C and 20 s at 72°C. The efficiency and specificity of all primers targeting bacterial sequences were assessed beforehand and only primer pairs with efficiencies higher than 1.95 were used. Absence of sample contamination by undigested genomic DNA was verified by RNA controls that did not undergo cDNA synthesis (“no RT controls”). Negative controls (blood or IST broth without spiked-in bacteria) were used similarly to ensure the absence of bacterial contamination or unintended primer binding to cDNA synthesised from human RNA.

Influence of Triton X-100 and saponin on the bacterial gene expression

E. coli CFT073 cells were grown in IST broth to the mid-logarithmic phase. 500 µl of the culture containing $\approx 2 \times 10^7$ c.f.u. were treated with 1 ml of RNAprotect for 15 min immediately. Another 500 µl of the same culture were treated with either 500 µl of 1 % [v/v] Triton X-100 in 500 mM cold carbonate buffer (pH 10.0) or 500 µl of 5 % [w/v] saponin in cold PBS buffer for 30 s. After centrifugation (5000x g for 3 min) a mix of 500 µl of IST broth and 1 ml of RNAprotect was added. RNA was extracted as described above and cDNA was synthesised from 600 ng of RNA. Real-time PCR analysis was performed for two reference genes (*gapA*, *adk*) and four genes (*rpoS*, *rpoH*, *pspA*, *cpxP*) that are known to respond to various stress conditions, such as a high pH, at a transcriptional level (Chung *et al.*, 2006; Danese & Silhavy, 1998; Darwin, 2005; Maurer *et al.*, 2005; Weiner *et al.*, 1991). Gene expression analysis was done using the Livak method (Livak & Schmittgen, 2001) and *gapA* for normalisation. Briefly, the difference (ΔC_q) in the C_q values between *gapA* and the gene of interest was calculated in the control (no lysis agent) and under the condition of interest (treatment with Triton X-100 or saponin).

220 Subsequently, the difference of both ΔC_q values was obtained, referred to as $\Delta\Delta C_q$. Error
221 bars represent the standard deviation of the ΔC_q values for each condition.

222 **Impact of spiked-in human RNA on real-time PCR detection of bacterial RNA**

223 All steps were carried out in a UV PCR cabinet to avoid contamination. Bacterial RNA
224 was extracted from IST grown cultures, human RNA was extracted from whole blood
225 (without bacteria) after mixing with RNAprotect. The initial concentrations of human and
226 bacterial RNA were determined with the Nanodrop ND-1000 instrument. Appropriate
227 amounts were obtained by serial dilution in nuclease free water. The combined species
228 were used for cDNA synthesis and qPCR as described above.

229 **Statistical analysis**

230 Statistical differences were identified using one-way ANOVA with *post-hoc* Holm-Sidak
231 tests. Differences were statistically significant at $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).
232 The degree of spread of the data is shown as standard deviation.

233

3. Results

RNA yield and integrity

In a first step, we compared the total RNA yields after RNA extraction and DNA digest. DNA accounted for about 30 % of the initially extracted nucleic acid. Blood with and without bacteria was either treated with RNAprotect immediately or lysed prior to the stabilisation.

The direct mixing of spiked blood and RNAprotect reagent without prior lysis of the human blood cells yielded about 40 - 50 % of the total bacterial RNA obtained from laboratory medium despite significant co-purification with human RNA (Fig. 1 (a)).

This method also led to a clotting of the blood sample. The resuspension of the pellet was tedious, time consuming and required some practice (i.e. potentially leading to user dependent variability). Additionally, remainders of the blood caused strong contamination of the silica columns used for nucleic acid purification. Lysis of the human blood cells prior to the stabilisation of the bacterial gene expression resulted in a decrease of co-purified human total RNA (Fig. 1 (a), grey bars). The effect was rather small for saponin, NH_4Cl and MoLYsis buffer CM, while treatment with Triton X-100 resulted in a strong reduction of the background below the limit of detection of the NanoDrop spectrophotometer. Upon mixing with the whole blood Triton X-100 degraded the blood cells within seconds and yielded about 60 % of the bacterial RNA compared to an extraction from IST broth. Similarly, saponin caused fast degradation of the human blood cells with only a small debris pellet remaining. However, a significant co-purification of human total RNA was still observed and the recovery rate for bacterial total RNA was 40 %. The A260/230 and

256 A260/280 ratios of the extracted RNA samples were around 2 for all tested conditions,
257 indicating the absence of high amounts of proteins or other contaminants (SI Fig. 1). If
258 the blood was first incubated with RNAprotect, then treated with one of the lysis agents,
259 no improvement of the RNA yield was observed. To the contrary, the RNA quality was
260 lower and the C_q values of bacterial mRNAs higher (data not shown).

261 To further investigate the reduction of the human RNA background after human blood cell
262 lysis we measured the remaining amount of human *β-actin* mRNA by means of qPCR
263 (Fig. 1 (b)). Samples directly stabilised with RNAprotect without prior treatment with a lysis
264 agent displayed the highest amount of *β-actin* mRNA. Treatment with Triton X-100
265 caused an increase of the C_q value by ≈12 units, corresponding to a 3 to 4 orders of
266 magnitude decrease in the concentration of *β-actin* mRNA. Interestingly, the amount of
267 detectable mRNA was reduced to a similar degree after lysis of the blood cells with
268 saponin, despite large amounts of co-purified total RNA (Fig. 1 (a)). Compared to RNA
269 stabilisation without prior application of a lysis agent, the amount of *β-actin* mRNA was
270 slightly but not significantly lower when the blood cells were lysed with NH₄Cl or MoYsis
271 buffer CM (p value 0.84 and 0.68, respectively).

272 The quality of the extracted RNA was assessed using capillary electrophoresis. The
273 electropherograms are shown in Fig. 2 and an overview of the resulting RINs (RNA
274 integrity numbers) is given in Fig. 1 (c). The electropherograms show two distinct peaks
275 for the bacterial 16S and 23S rRNA in all samples. Significant amounts of human 18S
276 rRNA and 28S rRNA were found in those samples that were stabilised immediately with
277 RNAprotect and those treated with NH₄Cl and MoYsis buffer CM (SI Fig. 2). No peaks
278 for human RNA were detected when the blood was lysed with saponin, although large

amounts of human total RNA were measured by the NanoDrop (Fig. 1 (a)). The observed RINs indicate high quality RNA ($RIN \geq 8$ (Imbeaud, 2005)) after blood cell lysis with Triton X-100 or saponin.

Finally, the implications of the used sample preparation method on specific mRNA quantities determined by real-time PCR were investigated by comparing the mRNA derived C_q values for three bacterial reference genes (*gapA*, *adk*, *gyrB*) (Fig. 1 (d)). The best C_q values, similar to those of the IST reference, were obtained when the blood was lysed with saponin. RNAprotect added directly to the sample or after lysis of the blood cells with Triton X-100 yielded C_q values about ≈ 1.5 units higher than the IST standard. However, standard deviations and the ΔC_q values between the genes remained similar compared to the reference. Only after blood cell lysis with NH_4Cl or MoYsis buffer CM we found a higher variability in the C_q value for *gapA*.

When bacterial RNA was stabilised with the PAXgene reagent total RNA yields were slightly lower than with RNAprotect and the C_q values for the investigated bacterial genes were notably higher (SI Table 1). Therefore, RNAprotect was used for the stabilisation of bacterial RNA throughout the rest of this study.

Influence of Triton X-100 and saponin on the bacterial gene expression

Since Triton X-100 and saponin were found to be the most powerful lysis agents for the human blood cells we investigated their effects on the bacterial gene expression by comparing C_q values for selected genes from treated and untreated samples extracted from IST broth.

When treated with Triton X-100 the C_q values for all investigated genes were significantly higher than for the untreated control despite equal input of total RNA (Fig. 3 (a)).

Total RNA yields and RINs were similar for treated and untreated samples, indicating that Triton X-100 did not affect the extraction process (SI Fig. 3). The average increase in the C_q values was dependent on the gene and varied between 0.56 (*cpxP*) and 1.33 (*pspA*). We hypothesised that the exposure to Triton X-100 leads to cell death. If the synthesis of new mRNA molecules is interrupted, their concentration is decreasing with a gene-specific half-life. To investigate the viability of the *E. coli* cells a viable cell count before and after incubation with Triton X-100 was performed. The recovery rate of viable bacteria after exposure to Triton X-100 for 3.5 min (time necessary for blood cell lysis and subsequent centrifugation) was ≈ 0.001 %. After incubation with saponin the recovery rate of viable bacteria was ≈ 100 %. In this case, C_q values were similar to the untreated control except for *rpoS* and *cpxP* (Fig. 3 (c)).

The treatment with either Triton X-100 or saponin was found to cause small but significant changes in the apparent observed expression of the investigated stress response genes (Fig. 3 (b), (d)). A 0.52-fold upregulation in the *cpxP* levels was observed for Triton X-100 and a 0.42-fold downregulation for saponin. Furthermore, a 0.26-fold downregulation of *rpoS* and a 0.58-fold upregulation of *rpoH* were observed after treatment with Triton X-100 and saponin, respectively.

Impact of spiked-in and co-purified human RNA on real-time PCR detection

In the next step, we investigated whether the direct application of RNaProtect to the blood sample without prior lysis of the blood cells was associated with the co-purification of PCR

inhibitors. As leucocyte DNA is a known inhibitor of PCR detection of bacterial DNA we hypothesised that leucocyte RNA could have a similar effect on bacterial RNA as both are transformed into cDNA before quantification. 0.05 ng of total bacterial RNA extracted from IST-grown cultures were spiked with serial dilutions of human RNA extracted from human whole blood without bacteria. Assuming the extraction efficiency from Fig. 1 (a) ($\approx 6 \mu\text{g}$ RNA from 2×10^7 cells) this amount of bacterial RNA corresponds to $\approx 2 \times 10^2$ c.f.u. For each mix cDNA synthesis was performed and the C_q values of a bacterial (*gapA*) and a human (*β -actin*) reference gene were investigated. The results are shown in Table 2.

With the addition of human RNA a decrease in the C_q value of *gapA* was observed which was significant for human RNA quantities between 4 and 400 ng. The lowest C_q values were obtained when the bacterial RNA sample was spiked with 40 ng of human RNA (corresponding to a human RNA concentration of 2 ng/ μl in the final cDNA synthesis reaction). The average decrease of the C_q value at this concentration was 2.69, corresponding to an apparent increase in the concentration of ≈ 6.5 -fold. No further improvement was observed at higher concentrations of human RNA. To the contrary, the C_q value for *gapA* increased when 400 ng of human total RNA were added. However, the signal was still significantly better compared to the unspiked control.

To investigate the impact of co-purified human RNA throughout the whole sample preparation and extraction process a much smaller amount ($\approx 10^4$ c.f.u.) of *E. coli* cells than in the previous experiments ($\approx 2 \times 10^7$ c.f.u.) was spiked into 0.5 ml of whole blood to drastically decrease the ratio between bacterial and human RNA. The RNA was either stabilised with RNAlprotect immediately or after lysis of the blood cells with Triton X-100 or saponin. Again, a sample extracted from IST broth served as a reference. As a

345 measure for the amount of bacterial mRNA the C_q values for *gapA*, *adk* and *gyrB* were
346 determined (Fig. 4).

347 The best signals were obtained if the bacterial RNA was extracted from whole blood after
348 direct stabilisation with RNAlprotect or after lysis of the blood cells with saponin, while the
349 extraction after blood cell lysis with Triton X-100 showed a lower efficacy, similar to the
350 IST reference. These results confirmed the findings of the spiking experiment (Table 2)
351 and demonstrate the beneficial impact of human RNA on the detection of small amounts
352 of bacterial RNA.

353

4. Discussion

RNA yield and integrity

The yields of bacterial total RNA and mRNA as well as the RNA integrities after RNA extraction from human whole blood were compared to a reference extraction from laboratory medium. The obtained RNA yields matched the numbers described in the literature. Bremer *et al.* (2008) determined a total RNA content of 214 $\mu\text{g}/10^9$ cells (corresponding to 4.28 $\mu\text{g}/2 \times 10^7$ cells) for *E. coli* growing with a doubling time of 24 min. (Bremer & Dennis, 2008) Our reference yields from IST broth were slightly higher ($\approx 6 \mu\text{g}/2 \times 10^7$ cells) but cells grew with a lower doubling time (19 min) and RNA yields will differ between strains. In whole blood, satisfactory results with respect to the total and mRNA yield were obtained when RNAprotect was added directly to the blood sample although large amounts of human RNA were co-purified. The method avoids the treatment with a lysis agent which might alter the gene expression, e.g. by activation of the bacterial stress response. However, the observed contamination of the silica column with debris might lead to clogging and reduction of the binding capacity when using larger volumes of blood. Huge amounts of co-purified human RNA can also interfere with some downstream applications such as gene expression measurements with microarrays (Di Cello *et al.*, 2005; La *et al.*, 2008) or RNA sequencing (Rienksma *et al.*, 2015). Triton X-100 was the most effective lysis agent and significantly reduced the amount of co-purified human total RNA and mRNA. After lysis of the human blood cells with saponin the amount of detectable human mRNA was considerably reduced, although there was still a significant remainder of human total RNA. An explanation for this observation might be the high degree of human RNA degradation indicated by the absence of distinct rRNA

peaks in the electropherogram (SI Fig. 2) as degraded RNA yields significantly higher C_q values (Fleige *et al.*, 2006). The main reason for the rather low efficacy of NH_4Cl and the MoLYsis lysis buffer might be the low temperature and the short exposure time (max. 30 s before centrifugation) used in our setup to keep the impact on the bacterial gene expression to a minimum. For the extraction of bacterial DNA from human whole blood the manual for the MoLYsis Basic5 kit recommends 5 min incubation with the lysis buffer at room temperature. (Molzym, 2014) For NH_4Cl other groups have used incubation times between 20 and 40 min (Brunck *et al.*, 2014) and found a low efficacy of the red blood cell lysis after short incubation (Boyd *et al.*, 2015).

The RNA quality is crucial for downstream applications because of its impact on the results obtained from gene expression measurements using real-time PCR (Fleige *et al.*, 2006; Schroeder *et al.*, 2006), microarrays (Thompson *et al.*, 2007) or RNA sequencing (Gallego Romero *et al.*, 2014). In this study, the RNA integrity number was satisfactory after lysis of the blood cells with either Triton X-100 or saponin, followed by stabilisation with RNAprotect. The RIN was lower for direct stabilisation with RNAprotect or after lysis blood cell lysis with NH_4Cl and MoLYsis buffer CM. However, these were the conditions with the highest amounts of co-purified human RNA which forms a background (Fig. 1 (a), SI Fig. 1) and might interfere with the RIN calculation.

Influence of Triton X-100 and saponin on the bacterial gene expression

We found an effect of both, Triton X-100 and saponin, on the bacterial gene expression. Saponin kept the cells alive and activated stress response genes (Fig. 3 (d)). Triton X-100 triggered cell death within the time frame of sample preparation. After cell death

mRNA molecules begin to degrade with a gene specific half-life of typically three to eight minutes (Bernstein *et al.*, 2002) until an RNA stabilising agent is added. Lysis with Triton X-100 will introduce a bias as mRNA species degrade with different half-lives. Additionally, a stress response might be induced before cell death. Especially, the observed upregulation of *cpxP* could be a response to the alkaline pH of the Triton X-100 solution. (Danese & Silhavy, 1998) However, for the genes investigated here the introduced bias was found to be small for both lysis agents. Consequently, all samples should be treated in the same way, i.e. extracted from whole blood using the same lysis agent and incubation times to minimise the impact of the sample preparation bias.

Impact of spiked-in and co-purified human RNA on real-time PCR detection

Initial experiments conducted here used bacterial concentrations of 10^7 c.f.u./ml, which is a common amount for gene expression studies in laboratory setups (Barczak *et al.*, 2012; Hedman *et al.*, 2012; Del Tordello *et al.*, 2012). However, in clinical bloodstream infections the bacterial concentrations often range between 10^0 and 10^3 c.f.u./ml (Dietzman *et al.*, 1974; Henry *et al.*, 1983; Werner *et al.*, 1967) drastically decreasing the ratio between bacterial and human RNA. As such low concentrations are relevant for diagnostic applications we conducted additional experiments with 10^4 c.f.u. (Fig. 4) and bacterial RNA corresponding to 10^2 c.f.u. (Table 2). It is important to note that the concentration of cells used in these experiments were defined by the concentration of mRNA per cell ($10^3 - 10^0$ copies/cell in *E. coli* (Taniguchi *et al.*, 2010)). To enhance the feasibility of transcription measurements for clinical application technical advancements for mRNA detection will be required. We found that moderate amounts of co-purified human RNA had a stabilising effect on small amounts of bacterial RNA. This seems contradictory to

observations made by other groups where a reduction of the human DNA background increased the sensitivity of bacterial DNA detection by means of qPCR (Loonen *et al.*, 2013; Zhou & Pollard, 2012), probably by reduction of mispriming and more efficient amplification (Song *et al.*, 2014). The stabilising effect of the human RNA observed here might be due to a relative reduction of adsorption of bacterial RNA to lab plastics as well as a relative protection of the target molecules from trace amounts of RNases which outweigh any potential negative effects. Thus, the co-purified human RNA acts as a carrier RNA. Similar protective effects of large amounts of foreign RNA have been described by other groups. (Andreasen *et al.*, 2010; Sanders *et al.*, 2011) Consequently, the direct addition of RNAprotect without prior lysis of the blood cells gave the best results for low numbers of bacterial cells with respect to signal intensity. Similar observations were made after lysis of the human blood cells with saponin. This protocol yielded highly degraded human RNA and facilitated the extraction process considerably by removal of cellular debris.

Conclusion

We successfully demonstrated the extraction of bacterial RNA from human whole blood and discussed the implications of the lysis of the human blood cells. A key conclusion of this study is that the selection of the most appropriate sample preparation method depends on factors such as number of bacteria, the impact of human RNA on downstream measurements or, potentially, sample volume. If gene expression experiments are performed with large numbers of bacteria grown in whole blood, the direct RNA stabilisation with RNAprotect is a good choice since the volume of blood is probably relatively small and a lysis agent that has a potential effect on the bacterial gene

expression is not required. However, it is not entirely clear whether RNAProtect works as effectively in blood as in laboratory medium. Furthermore, large amounts of human RNA are co-purified which may not be desirable for certain subsequent applications such as RNA sequencing or microarray experiments. If the experiment involves large amounts of human whole blood, lysis of the human blood cells with Triton X-100 or saponin may be useful as this facilitates the extraction considerably. Under these conditions the human RNA background is reduced significantly thus limiting the bias on downstream applications. This work will facilitate future fundamental studies to understand bacterial gene expression during blood stream infections as well as diagnostics developments.

Funding information

This work was funded by Medical Research Scotland (grant number PhD-619-2011). The stay of Klara Zwadlo and Simone Kraut at the University of Edinburgh was supported by an Erasmus plus EU mobility grant obtained by DKFZ.

Acknowledgements

The authors are grateful to Dr Medhat Khattar for valuable discussions of the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

Ethical statement

Human whole blood was obtained from a commercial source (Sera Laboratories International Ltd, West Sussex, United Kingdom). Donors were anonymised and informed about the use of their blood for research purposes. Therefore, no further ethics approval was required.

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689 **Table 1: Primers used for quantification of bacterial and human cDNA.**

Species	Gene description	Abbr.	Forward primer	Reverse primer
<i>E. coli</i> CFT073	glyceraldehyde-3-phosphate dehydrogenase	<i>gapA</i>	AGTTGACCTGAC CGTTCGTC	ACCCGCTTTAGCA TCGAACA
<i>E. coli</i> CFT073	adenylate kinase	<i>adk</i>	CTCCGAGCTGGG TAAACAAG	AGTTCGTCGGTGA CCAGTTT
<i>E. coli</i> CFT073	DNA gyrase subunit B	<i>gyrB</i>	GGCGTTTCCATTC GTCTG	CCGCCTTCATAGT GGAAGTG
<i>E. coli</i> CFT073	RNA polymerase factor sigma S	<i>rpoS</i>	ACGGGTAGGAGC CACCTTAT	TCCTCTTCGGCCA AATCGTT
<i>E. coli</i> CFT073	RNA polymerase factor sigma-32	<i>rpoH</i>	CCACCAAAGCGC AACGTAAA	AATCGTCGTCGGA AGACAGG
<i>E. coli</i> CFT073	phage shock protein PspA	<i>pspA</i>	TTTGCCGACATCG TGAATGC	ACGCACCAGTTTC TGTGGAT
<i>E. coli</i> CFT073	periplasmic repressor CpxP	<i>cpxP</i>	TGATGCGCATAGT TACCGCT	TCAGCAGCGTGGC TTAATGA
<i>Homo sapiens</i>	B-actin	-	TCACCCCACTGT GCCCATCTACGA (Barber <i>et al.</i> , 2005)	CAGCGGAACCGCT CATTGCCAATGG (Barber <i>et al.</i> , 2005)

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Table 2: C_q values for *gapA* (bacterial) and *β-actin* (human) after spiking of 0.05 ng of bacterial RNA with different amounts of human RNA extracted from whole blood. C_q values for *gapA* were compared to the unspiked control. n=3.

Spiked in human RNA [ng]	C _q <i>gapA</i> (± SDV)	p value	C _q <i>β-actin</i> (± SDV)
0	32.47 (± 0.81)	-	-
0.04	32.25 (± 0.51)	0.7137	34.73 (± 0.33)
0.4	32.04 (± 0.53)	0.4865	32.03 (± 0.85)
4	30.66 (± 0.55)	0.0326	26.95 (± 1.33)
40	29.78 (± 0.74)	0.0131	22.60 (± 0.85)
400	30.37 (± 0.86)	0.0364	19.55 (± 1.12)
neg. control*	-	-	19.39 (± 2.05)

* 400 ng of human RNA, 0 ng of bacterial RNA

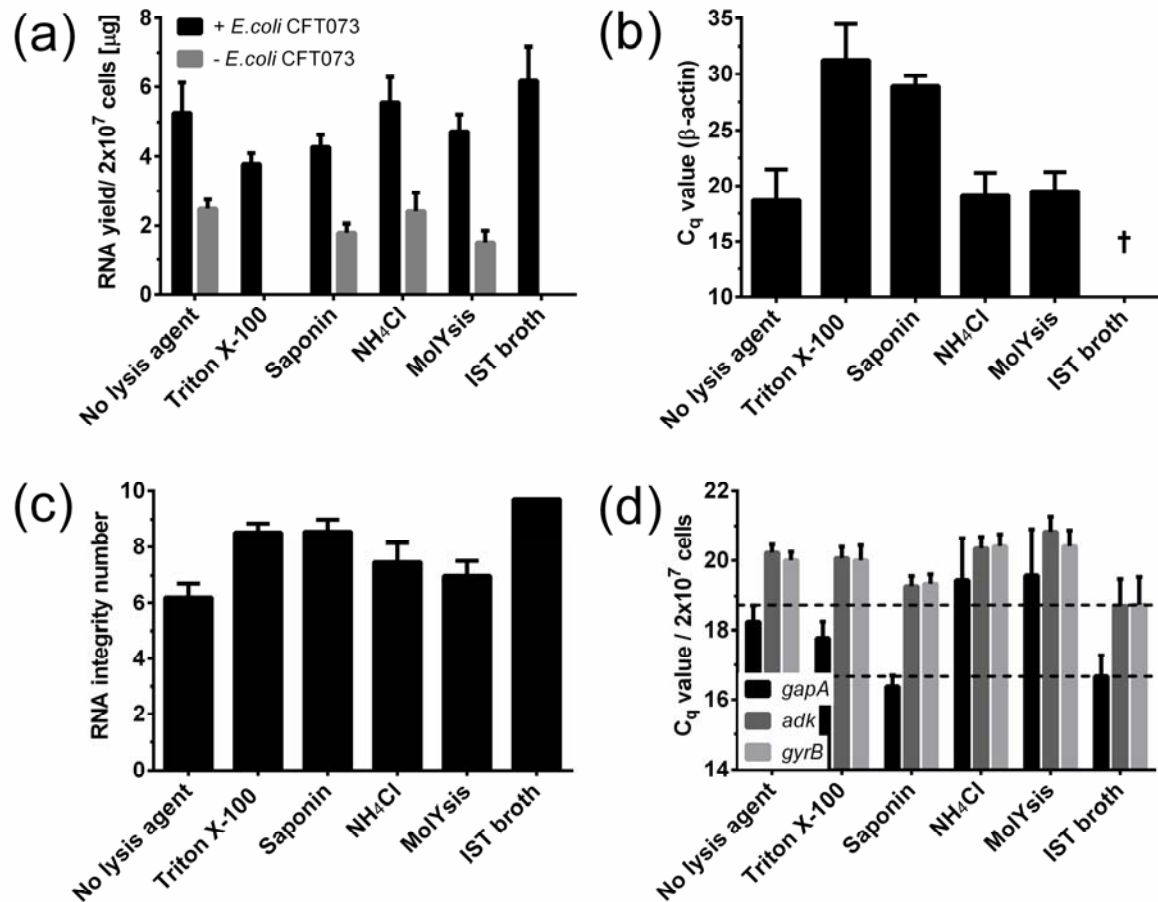
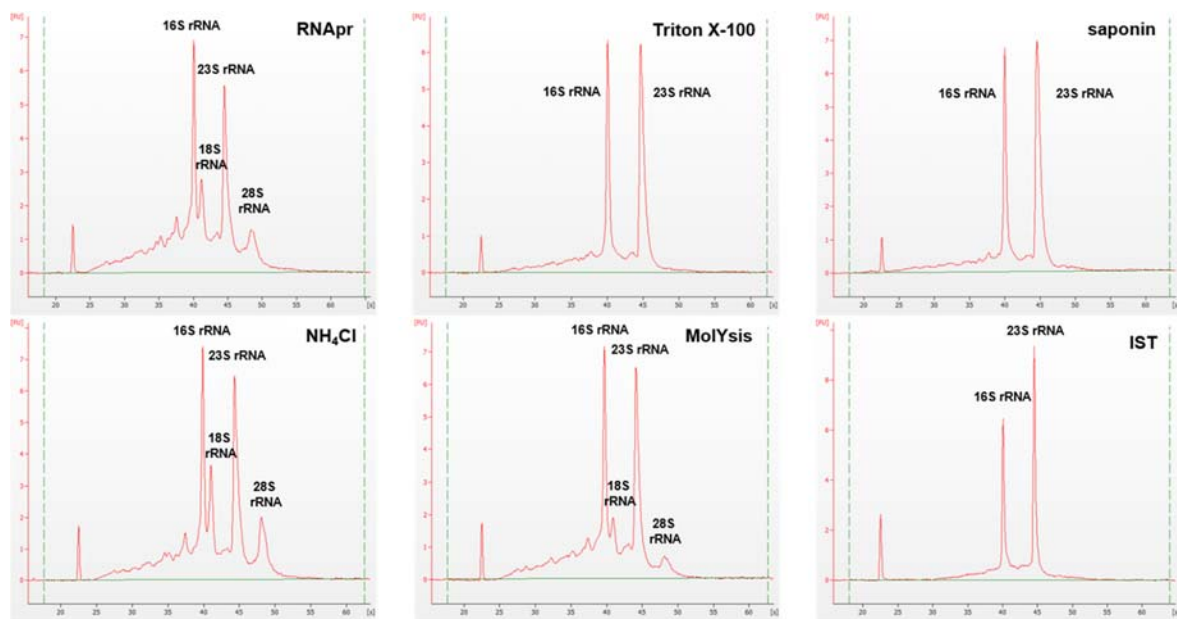


Fig. 1: Extraction of bacterial RNA from whole blood. (a) RNA yields after mixing whole blood with RNAprotect immediately ("no lysis agent") or after prior lysis of the blood cells with Triton X-100, saponin, NH₄Cl or MoYsis buffer CM. To obtain reference values extraction was also performed from IST broth after stabilisation with RNAprotect. Grey bars represent the RNA yield from 0.5 ml of human whole blood or IST broth alone, black bars represent the yield after spiking of 0.5 ml human whole blood or IST broth with $\approx 2 \times 10^7$ *E. coli* CFT073 bacteria. n = 6. (b) C_q values for human β -actin mRNA after treatment of 0.5 ml of whole blood or IST broth with RNAprotect directly, or after prior lysis of the blood cells. † indicates no C_q value (absence of β -actin mRNA). n = 3. (c) RNA integrity numbers (RINs) after stabilisation of the bacterial gene expression with RNAprotect immediately or after prior lysis of the blood cells. n = 6. (d) C_q values for *gapA*, *adk* and *gyrB* after stabilisation of the bacterial gene expression with RNAprotect immediately or after prior lysis of the blood cells. n = 6.



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710 **Fig. 2: Electropherograms of RNA obtained from whole blood samples containing *E. coli* CFT073**
 711 **bacteria.** RNA was extracted after adding RNAProtect to the sample immediately or after prior lysis of the
 712 human blood cells with Triton X-100, saponin, NH₄Cl or MolYsis buffer CM. The last electropherogram
 713 represents RNA extracted from IST broth after stabilisation with RNAProtect. 16S and 23S rRNA peaks
 714 represent bacterial rRNA, 18S and 28S rRNA peaks represent human rRNA.

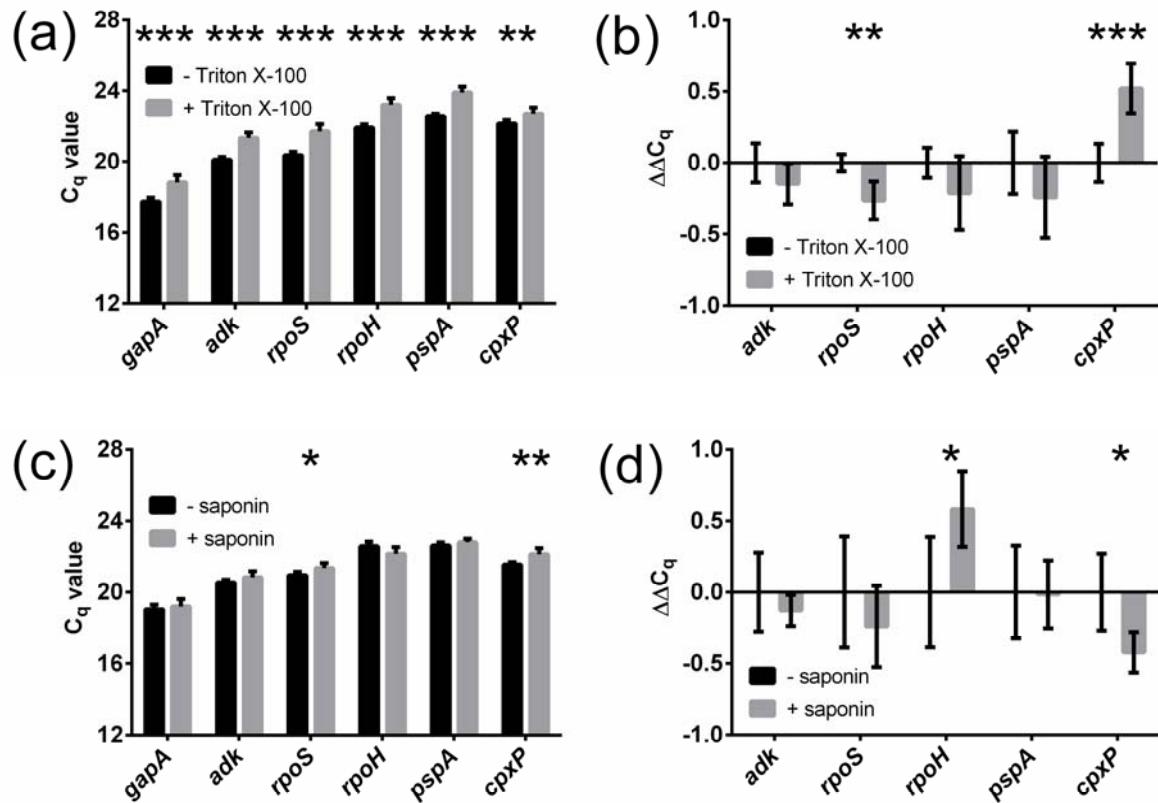


Fig. 3: Impact of lysis agents for human whole blood on the bacterial gene expression. (a), (c) Comparison of the C_q values of two reference genes (*gapA*, *adk*) and four genes of the bacterial stress response (*rpoS*, *rpoH*, *pspA*, *cpxP*) between cells treated with Triton X-100 (a) or saponin (c) and untreated controls. (b), (d) Observed fold changes after treatment with Triton X-100 (b) or saponin (d) after normalisation to *gapA*. $n = 5$.

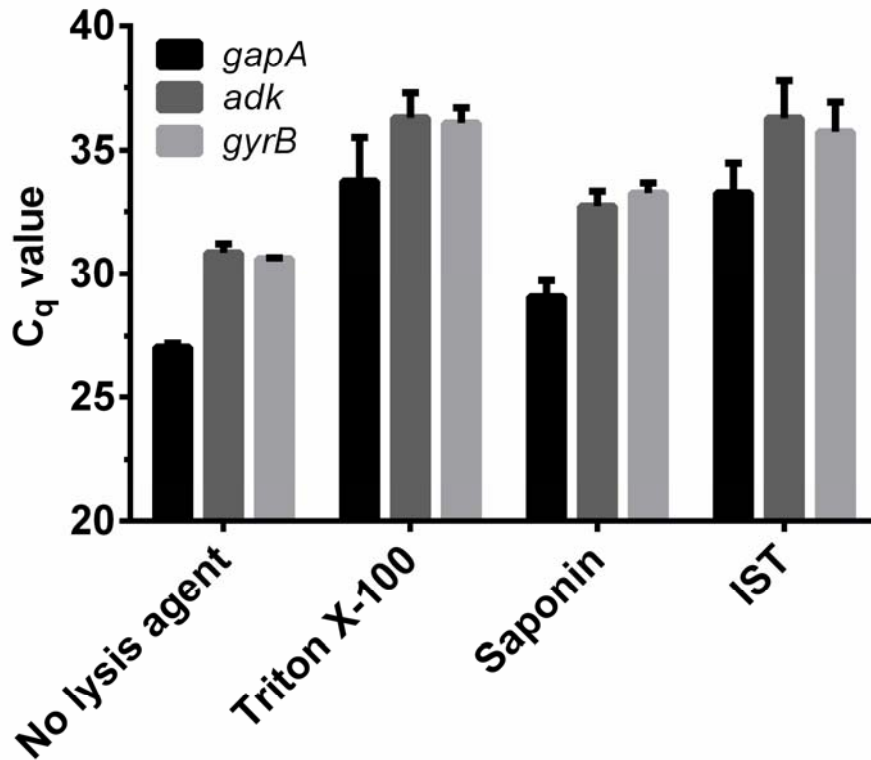
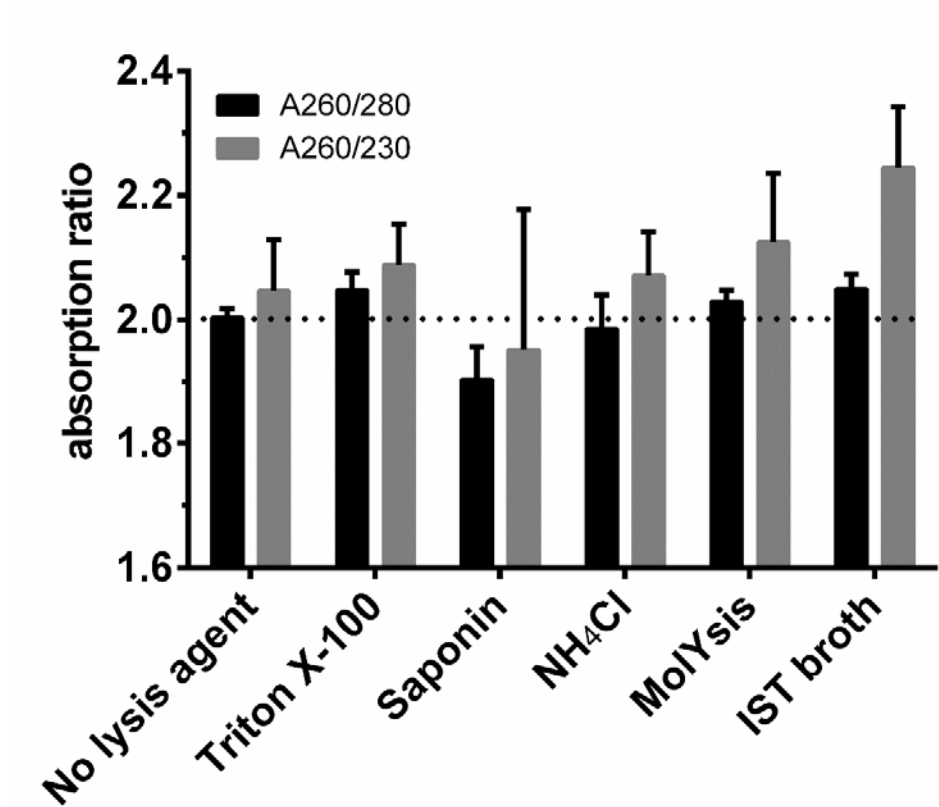


Fig. 4: Impact of co-purified human RNA on the detection of small amounts of bacterial RNA. mRNA abundance (C_q values) for *gapA*, *adk* and *gyrB* was measured after RNA extraction from 10^4 *E. coli* cells spiked into 0.5 ml of whole blood or IST broth. The RNA was extracted from IST broth after stabilisation of the gene expression with RNeasyprotect (standard), or from whole blood after direct stabilisation with RNeasyprotect or prior lysis of the blood cells with Triton X-100 or saponin. $n = 3$. No C_q values were obtained for the negative control (human whole blood without spiked-in bacteria).

1 Supplementary information

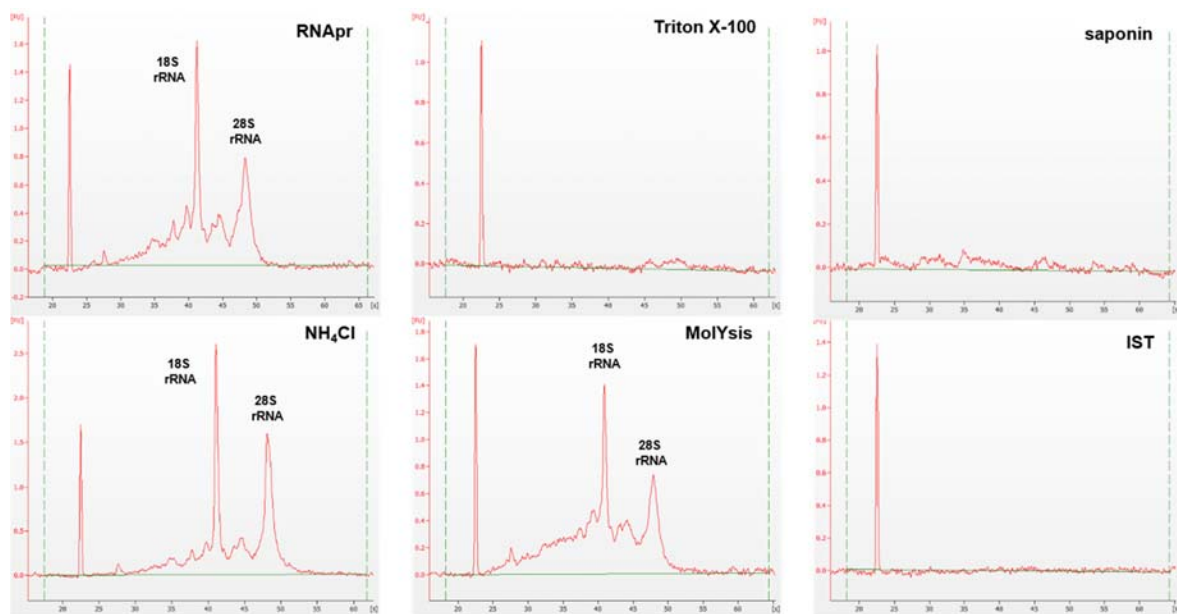
2 **SI Table 1: Extraction of bacterial RNA from human whole blood after stabilisation with PAXgene**
 3 **reagent. n = 6.**

Parameter	Value (\pm SDV)
Total RNA yield (0.5 ml of whole blood spiked with 2×10^7 c.f.u. of <i>E. coli</i> CFT073 bacteria) [ng]	2.47 (\pm 0.66)
Total RNA yield (0.5 ml of whole blood without spiked-in bacteria) [ng]	0.78 (\pm 0.12)
A260/280 ratio (0.5 ml of whole blood spiked with 2×10^7 c.f.u. of <i>E. coli</i> CFT073 bacteria)	1.98 (\pm 0.03)
A260/230 ratio (0.5 ml of whole blood spiked with 2×10^7 c.f.u. of <i>E. coli</i> CFT073 bacteria)	1.97 (\pm 0.07)
C _q value for human β -actin mRNA after RNA extraction from 0.5 ml of whole blood	20.17 (\pm 1.56)
RIN	8.1 (\pm 0.36)
C _q values for bacterial reference genes after RNA extraction from 0.5 ml of whole blood spiked with 2×10^7 c.f.u. of <i>E. coli</i> CFT073 bacteria	<i>gapA</i> : 19.43 (\pm 0.85) <i>adk</i> : 21.08 (\pm 0.41) <i>gyrB</i> : 21.02 (\pm 0.31)

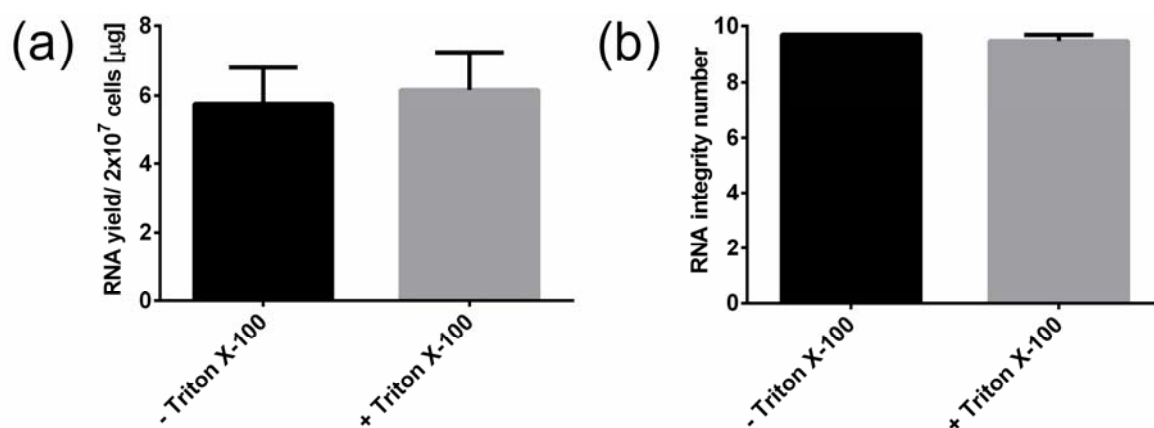


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5 SI Fig. 1: A260/280 and A260/230 ratios of RNA extracted from human whole blood with spiked-in
6 *E. coli* CFT073 bacteria. Extraction was either performed after mixing of whole blood with RNAprotect
7 immediately ("no lysis agent") or after prior lysis of the blood cells with Triton X-100, saponin, NH₄Cl or
8 MoYsis buffer CM. n = 6.



SI Fig. 2: Electropherograms of RNA obtained from whole blood samples without *E. coli* CFT073 bacteria. RNA was extracted after adding RNAProtect to the sample immediately or after prior lysis of the human blood cells with Triton X-100, saponin, NH₄Cl or MolYsis. The last electropherogram represents RNA extracted from IST broth after stabilisation with RNAProtect.



SI Fig. 3: Extraction of bacterial RNA from IST broth after treatment with Triton X-100. Yields are shown in (a) and RNA integrity numbers are shown in (b).